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Female color variation and male harassment in the polymorphic damselfly *Megalagrion calliphya*

Phoebe Cook

James Madison University

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Female color variation and male harassment in the polymorphic damselfly

Megalagrion calliphya

Phoebe Cook

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FACULTY COMMITTEE:

Committee Chair: Idelle A. Cooper

Committee Members:

Jonathan M. Brown

Reid N. Harris

Patrice M. Ludwig

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Abstract

Female dimorphism is commonly hypothesized to be a result of adaptations to male harassment. I tested whether polymorphic female coloration in the Hawaiian damselfly *Megalagrion calliphya* is under selection from male sexual harassment via two possible forms of negative frequency-dependent selection: the male mimicry and the learned mate recognition hypotheses. I measured male behavior toward tethered females at mating sites under naturally occurring conditions and found no evidence for either hypothesis. Harassment rates did not significantly differ between female morphs. One measure of morph frequency did predict harassment of all individuals, but this relationship was driven by a single population. I found no evidence that negative frequency-dependent selection contributes to the maintenance of polymorphism in this species. Future studies of female polymorphism should test other selective pressures which may act on polymorphism.

I. Introduction

Polymorphisms have long served as model systems for understanding how variation is maintained within populations (Gray and McKinnon 2007). The coexistence of distinct heritable morphs within a population can be maintained by balancing selective forces such as frequency-dependent selection or by gene flow between populations with divergent selection (Fisher 1930; Ford 1945; Endler 1977). In this study, I evaluated whether frequency-dependent selection could contribute to maintenance of female color polymorphism in a Hawaiian damselfly in which divergent selection across some populations is already implicated.

Sex-limited polymorphisms are those in which only one sex displays multiple morphs, and are commonly attributed to sexual selection (Hammers and Gossum 2008). Male-limited polymorphisms in many taxa are understood to be mating strategies that have evolved due to intrasexual competition over access to females (Dominey 1981; Forsyth and Alcock 1990; Gross 1991; Saetre and Slagsvold 1996). Female-limited polymorphisms, on the other hand, are thought to be the result of intersexual conflict over mating rate (Wellenreuther et al. 2014). High mating rates may increase male fitness, but because females can fertilize their clutch with a single copulation, additional matings or harassment may have a fitness cost (Bateman 1948; Trivers 1972; Sirot and Brockmann 2001). Female-limited dimorphism has been studied most commonly in damselflies, where it has been described in more than 100 species (Fincke et al. 2005). In these species, one female morph (called the *andromorph*) is similar to the male in coloration, while one or more other morphs (*gynomorphs*) are distinct from the male.

Multiple hypotheses have been proposed for how selection from male mating harassment could drive the evolution of such polymorphisms, but the two hypotheses given the most attention are the male mimicry hypothesis and the learned mate recognition hypothesis. The male mimicry (MM) hypothesis states that andromorphs escape harassment by mimicking the appearance of males (Robertson 1985). If males do not harass individuals who appear to be male, andromorphs encounter lower rates of harassment than gynomorphs. This strategy is expected to be successful when andromorphs are rare, but as mimics become more common relative to the males they imitate, the benefits of mimicry become reduced because males harass all male-like individuals more (Fincke et al. 2005; Ting et al. 2009; Xu and Fincke 2011). Under this hypothesis, the rate of harassment towards gynomorphs is not expected to vary with morph frequency (Ting et al. 2009) because gynomorphs are always recognized as potential mates. The predictions of the MM hypothesis, therefore, are that harassment of andromorphs varies depending on population morph frequencies while harassment of gynomorphs is constant across populations (Figure 1). The learned mate recognition (LMR) hypothesis predicts that harassment of both morphs will be negatively frequency-dependent (Miller and Fincke 1999; Fincke 2004). If males learn to recognize mates based on which female morph is common in their population, harassment on each morph will be greatest when that morph is common (Figure 1). Both hypotheses assume that harassment carries a fitness cost to the female.

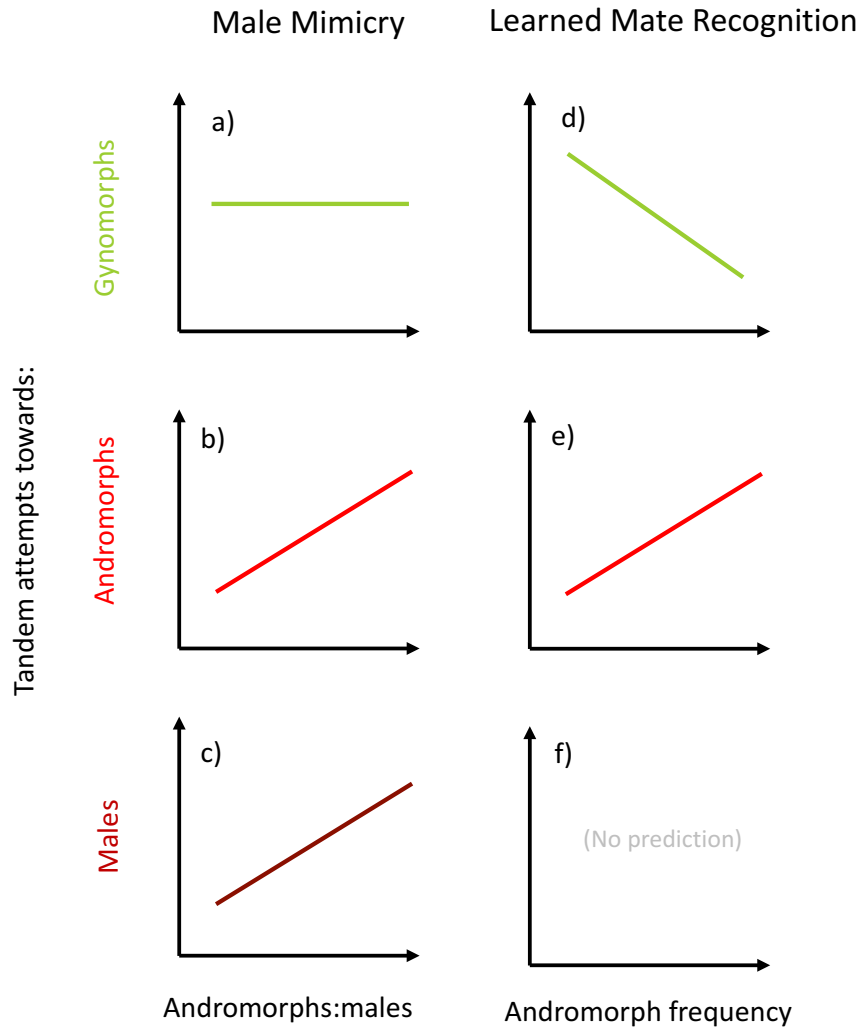


Figure 1. Predictions of the two harassment hypotheses, using tandem attempts as an example sexual behavior. Under the male mimicry hypothesis (a-c), rates of tandem attempts toward andromorphs and males are predicted to increase with the ratio of andromorphs to males (“mimics to models”). Under the learned mate recognition hypothesis (d-f), harassment rates of gynomorphs and andromorphs are expected to be negatively correlated with frequency. Figure adapted from Ting et al. 2009.

In addition to negative frequency-dependent selection, female polymorphisms can also be maintained by variation in selection both between and within populations (Huxley et al. 1955; Endler 1977; Galeotti et al. 2003). If selection varies between populations,

with each morph having a selective advantage in some areas, gene flow could lead to a range of population morph frequencies (Endler 1977). In some damselfly species, these are correlated with biotic variables such as temperature, but the role of ecological selection is unclear (Hammers and Gossum 2008; Sánchez-Guillén et al. 2011; Takahashi et al. 2011; Iserbyt et al. 2012). Selection may also vary within a population. If morphs exploit different ecological niches, both could be maintained (Van Gossum et al. 2008).

The various hypotheses are not necessarily mutually exclusive. Both spatially-varying ecological selection and frequency-dependent sexual selection may operate on color in a single system. Without testing both sexual and ecological selection hypotheses, conclusions as to which is primarily responsible cannot be drawn (Svensson 2017).

Previous research found support for an ecological selection hypothesis in the female-polymorphic Hawaiian *Megalagrion calliphya* damselflies (Cooper 2010; Cooper et al. 2016), and here I test the sexual selection hypotheses. I measured male behavior toward tethered gynomorphs, andromorphs, and males at mating sites under naturally occurring conditions, similar to tests of male harassment in other studies (e.g. Cordero et al. 1998; Miller and Fincke 1999; Andrés and Sánchez-Guillén 2002; Ting et al. 2009). I conducted the behavioral observations at five populations that vary in morph frequency. The predictions of the sexual selection hypotheses are that male *M. calliphya* would direct more harassment at gynomorphs (MM hypothesis) or at the more common morph in a population (LMR hypothesis). Previous field observations do not provide support for either the male mimicry or learned mate recognition hypothesis: Cooper found no overall difference in mating rates between morphs and no relationship between the mating rate of each morph and its frequency in the population. However, these observations did not

manipulate female presence at the mating habitat. If females escape unwanted interactions by leaving the area, observational studies can underestimate the harassment females would experience if present and the costs they may incur by fleeing (Van Gossum et al. 2001; Fincke et al. 2007).

II. Materials and Methods

Natural history

The endemic Hawaiian damselfly *Megalagrion calliphya* lives near intermittent streams and upland bogs. Males defend territories around the open water where females oviposit, while females spend most of their time away from the water and are usually found near the water only when mating. Males initiate mating by clasping the female by the thorax using abdominal appendages. The pair may stay in this position, called tandem formation, for some time before the female chooses to copulate by moving her abdomen forward to make contact with the genitals of the male. The pair will then perch just above open water and the female will oviposit. Other males may approach the pair and attempt to drive off the male.

The body color of males and andromorphs is red, and gynomorphs are green. The basis of this variation is unknown, but in other damselfly species female-limited color polymorphisms are determined by one or two autosomal loci (Johnson 1964, 1966, Cordero 1990, Andrés and Cordero 1999, Sánchez-Guillén et al. 2005, Takahashi et al. 2010). In *M. calliphya*, color does not change between red and green over the lifespan of individual adults (unpublished data). A previous study found that the frequency of andromorphs varied between populations on Hawaii Island from almost 0% to 100% (Cooper 2010).

Sex and morph ratios

All work reported here took place in five *M. calliphya* populations on the windward slope of Mauna Loa on Hawaii Island. I measured sex and morph ratios for each of the five populations between June 18 and August 8, 2015 (Table 1). Unmarked

adults were netted in multiple passes through the mating habitat between 0800 and 1400 hours on days that it was not raining. Captured individuals were identified as male, andromorphic female, or gynomorphic female; marked on the wing with a unique number in indelible ink; and scanned using a portable flatbed scanner before release. Scans of damselflies laid with their wings immobilized by microscope slides provide high-resolution images of the lateral thorax at a consistent angle, lighting, and scale with minimal handling (Figure 1). Interclass correlation coefficients calculated in the package ICC (Wolak et al. 2012) for repeated measurements of 136 damselflies in a pilot trial showed that the results of this method were highly repeatable (hue: ICC = 0.998; saturation: ICC = 0.978, brightness: ICC 0.909). This suggests that this method does provide reliable color data. I also reviewed scans to confirm field identifications of sex and morph.

Table 1. Elevation and demographic information for the five populations, including the total number of gynomorph females (G), andromorph females (A), and males (M) captured.

Population	Elevation	G, A, M	Andromorph freq. A/(G+A)	Male freq. M/(M+A+G)	Mimics:Models A:M
Ninole	646 m	11, 0, 101	0.00	0.90	0
Portuguese	792 m	63, 6, 380	0.09	0.85	0.02
Waiaele	853 m	91, 70, 451	0.43	0.74	0.16
UMHR	1036 m	50, 28, 426	0.36	0.85	0.07
Kulani	1676 m	5, 31, 289	0.86	0.89	0.10

Color measurements

In order to quantify color variation as a continuous variable, I measured the thorax color of each focal individual from the scans in Adobe Photoshop CS6 using previously established methods (Cooper 2010; Cooper et al. 2016). Color was averaged over an area

of the lateral thorax delimited by consistently identifiable physical landmarks. Hue, saturation, and brightness (HSB) values or hex codes were measured directly in Photoshop, and then hex codes could be translated into HSB values using the R package *colorspace* (Ihaka et al. 2016).

Behavior trials

I measured harassment of tethered focal individuals at the five populations during 64 trials between June 28 and July 11, 2015. Focal individuals were collected from the mid-elevation populations Waiaele and Upper Mountain House Road where both morphs are abundant, held for less than 48 hours, and brought to the study sites (including the populations of origin) in coolers packed with ice and damp paper towels to reduce the stress of transport. Within each trial, all focal individuals originated from the same population.

In each trial the focal individuals included one gynomorph, one andromorph, and one male that I spaced equally at a distance of approximately 30 cm around the edge of a pool and secured to vegetation or rocks using Beacon Fabri-Tac[®] adhesive on their legs. This method allowed for natural movement of the head, abdomen, and wings but not for departure from the mating site. The use of tethered females is common in studies of harassment in Odonates (e.g. Cordero et al. 1998; Miller and Fincke 1999; Andrés and Sánchez-Guillén 2002; Ting et al. 2009). It is important to note that I did not actively present tethered females to males, as is done in studies where the focus is on male mate preference.

A stationary observer at least one meter away from the pool recorded local male behavior towards all focal individuals for one hour during each trial. For each focal

individual, I recorded how often local males came within 10 cm, as well as the incidence and duration of males hovering without contact, contacts, tandem attempts, tandem formations, and attempted or successful takeover of tandems by other males. Tandem attempts, formations, and take-overs were classified as sexual behaviors (Fincke 1994). I also recorded when focal individuals responded to tandem attempts with a rejection display (Preston-Mafham and Preston-Mafham 1993; Fincke 1997). I did not control for repeated interactions by local males, as they were not marked individually and could not be identified.

Each day of the study, behavioral trials were initiated once mating pairs were seen at the site and were run throughout the peak mating period, between 0830 and 1330 hours. Trials were set up only around pools where at least one territorial male had been observed, which indicated that the pool was suitable mating habitat. These conditions prevented artificially low harassment rates due to sampling during poor weather or in unused territory.

Statistical analysis

To describe color variation, HSB values were compared between males, andromorphs, and gynomorphs using Kruskal-Wallis tests, followed by post-hoc Nemenyi tests in the R package PMCMR (Pohlert 2014). Hue is a circular variable, with 0 and 360 representing the same value, and male thorax hues fall on either side of that division. In order to make hue continuous, values near 360 were recoded as negative numbers by subtracting 360, following Cooper (2016). I recoded the hue values of two males, from 358 and 359 to -2 and -1. All other hues fell between 0 and 80.

Male harassment toward female focal individuals was quantified using the number of sexual interactions and the total duration of sexual interactions (tandem attempts, formations, and takeovers). The number of sexual interactions was analyzed using a negative binomial regression suitable for over-dispersed count data in the R package MASS (Venables and Ripley 2002). The predictors were individual focal morph, andromorph frequency, and their interaction. The individual type term tests for an overall difference in harassment levels between the morphs, morph frequency tests for differences in harassment rates between sites, and the interaction term tests for frequency-dependence. Time spent in sexual interactions was log-transformed and analyzed using a linear model with the same predictor variables.

In order to test the predictions of the male mimicry and learned mate recognition models (Fig. 1), the number of sexual interactions directed at males, gynomorphs, and andromorphs was modeled using both andromorph frequency relative to gynomorphs and relative to males (Ting et al. 2009). Because I have one of each frequency measurement for each site, both could not be included in the same model. All statistical analyses were performed in R 3.2.4 (R Core Team 2016).

III. Results

Quantitative color variation

In the mid-elevation populations in which I collected focal individuals, hue, saturation, and brightness differ significantly between gynomorph females, andromorph females, and males (Kruskal-Wallis $X^2 = 136.71$, $df = 2$, $p\text{-value} < 0.0001$ for hue; $X^2 = 136.49$, $df = 2$, $p\text{-value} < 0.0001$ for saturation; and $X^2 = 12.774$, $df = 2$, $p\text{-value} = 0.0017$ for brightness). Gynomorphs differ significantly from males in all measured aspects of color; they are less red, less saturated, and less bright (Nemenyi post-hoc tests: $p < 0.0001$, $p < 0.0001$, and $p = 0.0375$, respectively). Andromorphs are intermediate to males and gynomorphs in all three measured aspects of color. Andromorphs are significantly less red and less saturated than males, but are not significantly different from males in brightness (Nemenyi post-hoc tests: $p = 0.0029$, $p = 0.001$, $p = 0.5590$).

Predictors of harassment

Morph was not a significant predictor of either number of sexual interactions or total duration of sexual interactions (Tables 2 and 3). Andromorph frequency relative to gynomorphs was a significant predictor of both, with higher levels of harassment of all groups associated with higher andromorph frequencies relative to gynomorphs. This relationship was driven by the highest-elevation population, Kulani. When this population was removed from the analysis, andromorph frequency no longer predicted harassment (general linear model, $p = 0.37$; negative binomial regression $p = 0.56$). The ratio of andromorphs to males did not predict either number or duration of sexual interactions. The interaction term between morph and frequency was not significant in any model.

Table 2: General linear model of time spent in sexual interactions. Significant values are indicated with an asterisk.

	df	F	p		df	F	p
Morph	1	5.91	0.27	Morph	1	1.18	0.28
A/G Frequency	1	29.16	0.02*	A/M Frequency	1	2.27	0.13
Morph x Frequency	1	9.56	0.16	Morph x Frequency	1	3.18	0.08

Table 3: Negative binomial regression models of number of interactions.

	df	X ²	p		df	X ²	p
Morph	1	0.52	0.47	Morph	1	0.47	0.49
A/G Frequency	1	6.46	0.01*	A/M Frequency	1	1.72	0.19
Morph x Frequency	1	1.05	0.31	Morph x Frequency	1	1.52	0.22

There was wide variation in both measures of harassment (Figures 3 and 4). The outliers in duration represent interactions in which males clasped the focal individuals in tandem and did not release them for long periods of time. There were also large numbers of zeroes in both the number and duration of interactions. Of 128 females, 101 had no sexual interactions with males over the course of an hour, even though males were active in the area. In 49 of those cases, males approached within 10 cm of the female, but did not attempt to mate with her. There were only 45 successful tandem events in 128 damselfly-hours of trial time, for an average of 0.35 tandems per hour. In order to be sure that these zeroes were not altering the results, I reran all models after discarding individuals in trials with no interactions, then individuals with no interactions. Results were not qualitatively impacted, with the exception that the interaction between morph and A:M ratio predicted number of interactions (see Appendix). Coefficients were of the opposite sign than would be expected under negative frequency-dependence.

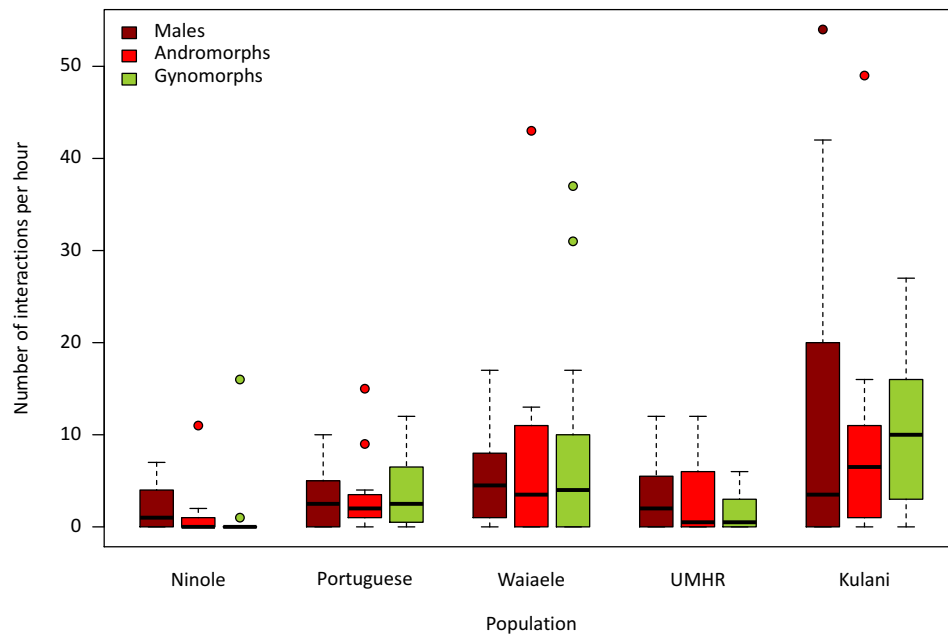


Figure 2. Number of total interactions experienced by males, andromorphs, and gynomorphs in each population. Populations are ordered by elevation.

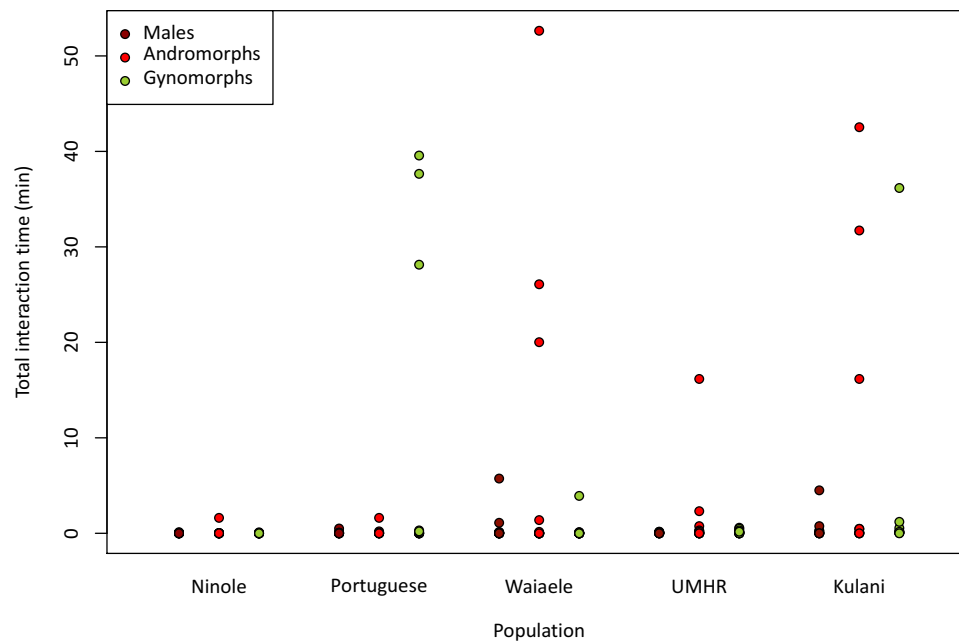


Figure 3. Time spent in interactions by males, andromorphs, and gynomorphs in each population. Populations are ordered by elevation. Because of high numbers of zeroes and extreme outliers, points are drawn rather than boxplots.

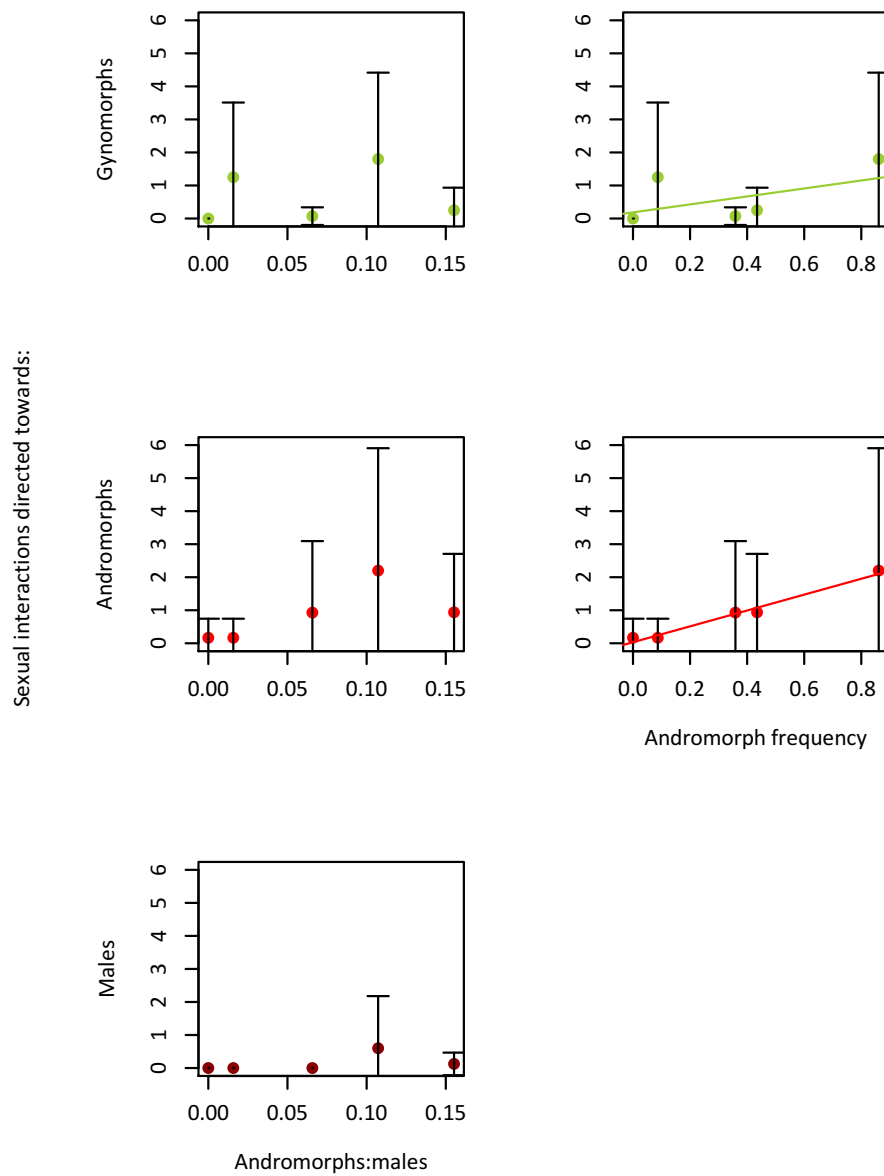


Figure 4. Sexual interactions directed toward focal individuals plotted against andromorph:males ratios and andromorph frequencies, following Figure 1. Points represent mean numbers of tandem attempts per population, bars represent 1 SD. Regression lines are included when the frequency term in the model of number of sexual interactions was significant.

IV. Discussion

I found no evidence of selection from male harassment on female color in this system. Although andromorphs differ significantly from gynomorphs in all measured components of color variation, there was no difference in harassment rates between morphs. There were also no differences in frequency-dependence between morphs. Our results do not support either the male mimicry or learned mate recognition hypotheses.

Andromorph frequency was the only significant predictor of harassment experienced, as measured by either number of interactions or their duration (Tables 2 and 3). The significance was driven by a single population, Kulani; the population with the highest andromorph frequency had higher harassment of all focal individuals. This result out of context might suggest that andromorphism is an escape from intense harassment, but gynomorphs and andromorphs were harassed at equal rates at all sites, including the majority-andromorph population. Since each population had a single value for andromorph frequency, frequency is confounded with all differences between sites, including ecological differences that may affect harassment rate. Unlike the four lower-elevation intermittent streams, this site is a high-altitude bog. Weather is highly variable because of a trade wind inversion at this elevation that can trap moisture in clouds, which could lead to less suitable periods of fine weather for mating. There is also limited surface water available for territory defense and oviposition, despite abundant underground aquatic habitat to support a large population size, which may lead to more interactions between adults. These conditions at high elevation may concentrate the adult interactions in time and space.

The rates of harassment experienced did not follow any of the patterns predicted by either male mimicry or learned mate recognition hypotheses (Ting et al. 2009) (Figure 4). The andromorphs:males ratio did not predict the number of sexual interactions directed at andromorphs, as predicted by the male mimicry hypothesis. Andromorph frequency did predict the number of interactions, consistent with the learned mate recognition hypothesis, but this relationship was the same for both female morphs. Harassment rates of andromorphs and gynomorphs were both positively correlated with andromorph frequency, rather than positively correlated in andromorphs and negatively in gynomorphs. This trend was again driven by the highest-elevation population, and without that population no pattern seen in the other four sites. Finally, I note that one of the five populations is monomorphic, which is not expected if morphs are maintained by negative frequency-dependent selection (Svensson 2017).

Strikingly, I detected low rates of harassment across populations, morphs, and sexes. Since females were restrained for the behavioral observation trials, it is possible that our experimental setup would not detect harassment if males are more likely to recognize females in flight. However, this possibility applies to all studies of tethered damselflies, many of which show high rates of harassment. Our average of 0.35 tandems per hour means that the average female at the mating site is clasped in tandem just under once every three hours. These rates are dramatically higher in other studies on *Enallagma hageni*, *Ischnura elegans*, and *Nehalennia irene*, in which males search for mates and scramble to mate with females when they find them, leading to intense harassment of females (Fincke et al. 2007; Xu and Fincke 2011). Males of *Megalagrion* species, by contrast, are territorial (Moore 1983) and therefore females may be able to avoid

unwanted interactions much more easily by simply leaving the mating territory.

Harassment seems to be a much weaker selective pressure in this species than in other damselflies, potentially because of different mating systems. An interesting future comparison would be to test these hypotheses in more territorial species and examine the effect of mating system and harassment on a larger taxonomic scale.

This study found no evidence of sexual harassment on female polymorphism in *Megalagrion calliphya*, suggesting that this polymorphism is maintained by other selective forces. In the damselfly genus *Megalagrion*, there is evidence that female polymorphism is under ecological selection from abiotic factors which vary within and between populations. Color variation is correlated with canopy cover; green gynomorphic females are prevalent in low-elevation habitats with dense canopy cover, and red andromorphic females are common at high-elevation populations with sparse vegetation (Cooper 2010; Cooper et al. 2016). Red males defend territories in exposed habitat throughout their elevational range. The red pigment function may be antioxidant defense against oxidative damage from UV damage in exposed habitat, in both males and females (Cooper 2010). Green females may have an advantage in shaded habitat due to a physiological cost or a predation risk of the red pigmentation. A spatial pattern of selection, combined with gene flow, could lead to a cline in morph frequencies (Endler 1977).

Debate over the relative contributions of ecological and sexual selection to the evolution of dimorphisms dates back to Darwin and Wallace (Darwin 1871; Wallace 1889; Kottler, 1980; (Kottler 1980; Andersson 1994; Punzalan and Hosken 2010). Decades of work on female polymorphism have mostly focused on sexual conflict

hypotheses. Recently, there have been more calls to consider multiple selective forces which may act on polymorphic traits either in combination or separately (Sánchez-Guillén et al. 2011; Iserbyt et al. 2012). Future studies of female polymorphism should test ecological as well as sexual selective forces. The same patterns of polymorphism in different species may be the result of different processes, especially when the species differ in ecology and natural history.

Appendix – Supplementary Materials

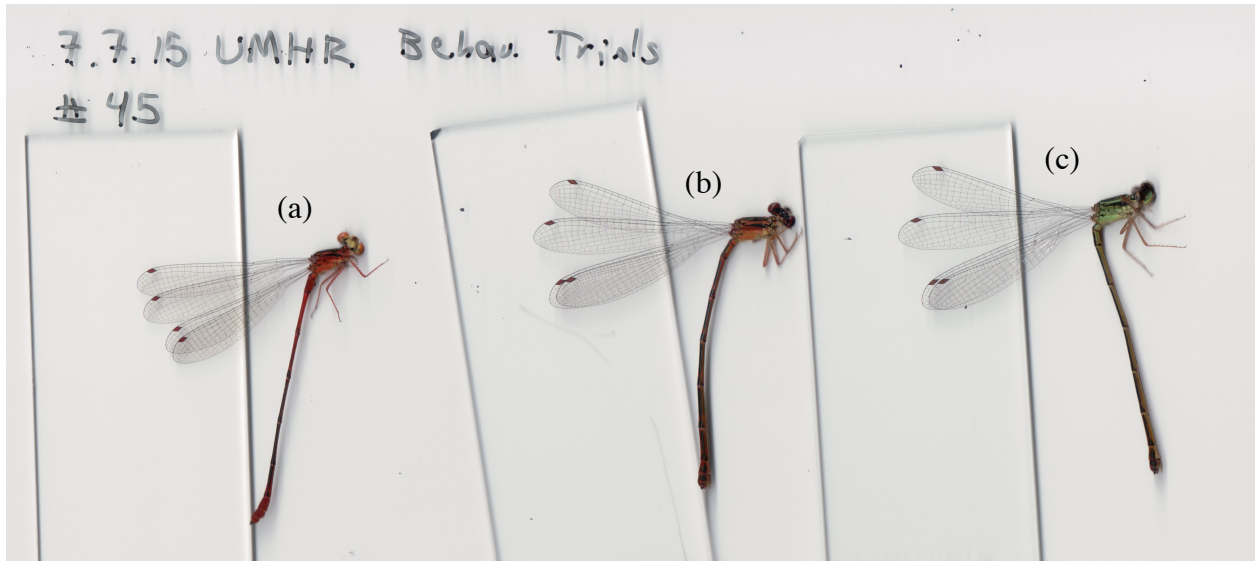


Fig. S1. Scan of the (a) male, (b) andromorph, and (c) gynomorph female in one trial.

Table S1: General linear model of time spent in sexual interactions, without zeroes.

	df	F	p		df	F	p
Morph	1	6.51	0.34	Morph	1	6.51	0.33
A/G Frequency	1	20.67	0.09	A/M Frequency	1	19.06	0.10
Morph x Frequency	1	18.56	0.11	Morph x Frequency	1	38.20	0.02*

Table S2: General linear model of time spent in sexual interactions, without individuals from trials with no interactions.

	df	F	p		df	F	p
Morph	1	6.64	0.27	Morph	1	6.51	0.33
A/G Frequency	1	31.38	0.02*	A/M Frequency	1	19.06	0.10
Morph x Frequency	1	10.62	0.16	Morph x Frequency	1	38.20	0.02*

Table S3: Negative binomial regression models of number of interactions, without zeroes.

	df	X ²	p		df	X ²	p
Morph	1	0.35	0.56	Morph	1	0.36	0.55
A/G Frequency	1	1.80	0.18	A/M Frequency	1	4.27	0.04*
Morph x Frequency	1	2.85	0.09	Morph x Frequency	1	1.61	0.20

Table S4: Negative binomial regression models of number of interactions, without zeroes.

	df	X ²	p		df	X ²	p
Morph	1	0.54	0.46	Morph	1	0.48	0.48
A/G Frequency	1	6.46	0.01*	A/M Frequency	1	1.75	0.19
Morph x Frequency	1	1.06	0.30	Morph x Frequency	1	1.58	0.21

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